

## ALTERATION OF AMINO ACID COMPOSITIONS IN SEEDS

Rudolf Jung  
Larry R. Beach  
Virginia M. Dress  
A. Gururaj Rao  
Jerome P. Ranch  
David S. Ertl  
Regina K. Higgins

### BACKGROUND OF THE INVENTION

Feed formulations based on crop plants must typically be supplemented with specific amino acids to provide animals with essential nutrients which are necessary for their growth. This supplementation is necessary because, in general, crop plants contain low proportions of several amino acids which are essential for, and cannot be synthesized by, monogastric animals.

The seeds of crop plants contain different classes of seed proteins. The amino acid composition of these seeds reflects the composition of the prevalent classes of proteins. Amino acid limitations are usually due to amino acid deficiencies of these prevalent protein classes.

Among the amino acids necessary for animal nutrition, those that are of limited availability in crop plants include methionine, lysine, and threonine. Attempts to increase the levels of these amino acids by breeding, mutant selection, and/or changing the composition of the storage proteins accumulated in the seeds of crop plants, have met with limited success, or were accompanied by a loss in yield.

For example, although seeds of corn plants containing a mutant transcription factor, (opaque 2), or a mutant  $\alpha$ -zein gene, (floury 2), exhibit elevated levels of total and bound lysine, there is an altered seed endosperm structure which is more susceptible to damage and pests. Significant yield losses are also typical.

An alternative means to enhance levels of free amino acids in a crop plant is the modification of amino acid biosynthesis in the plant. The introduction of a feedback-regulation-insensitive dihydrodipicolinic acid synthase ("DHDPS") gene, which encodes an enzyme that catalyzes the first reaction unique to the lysine biosynthetic pathway, into plants has resulted in an increase in the levels of free lysine in the leaves and seeds of those plants. An increase in the levels of free lysine in the embryo results in reduced amount of oil in the seed. Further free lysine can be lost during the wet milling process reducing the feed value of the gluten product of the process.

The expression of the *lysC* gene, which encodes a mutant bacterial aspartate kinase that is desensitized to feedback inhibition by lysine and threonine, from a seed-specific promoter in tobacco plants, has resulted in an increase in methionine and threonine biosynthesis in the seeds of those plants. See Karchi, *et al.*; The Plant J.; Vol. 3; p. 721; (1993). However, expression of the *lysC* gene results in only a 6-7% increase in the level of total threonine or methionine in the seed. The expression of the *lysC* gene in seeds has a minimal impact on the nutritional value of those seeds and, thus, supplementation of feed containing *lysC* transgenic seeds with amino acids, such as methionine and threonine, is still required.

There are additional molecular genetic strategies available for enhancing the amino acid quality of plant proteins. Each involves molecular manipulation of plant genes and the generation of transgenic plants.

Protein sequence modification involves the identification of a gene encoding a major protein, preferably a storage protein, as the target for modification to contain more codons of essential amino acids. An important aspect of this approach is to be

able to select a region of the protein that can be modified without affecting the overall structure, stability, function, and other cellular and nutritional properties of the protein.

The development of DNA synthesis technology allows the design and  
5 synthesis of a gene encoding a new protein with desirable essential amino acid compositions. For example, researchers have synthesized a 292-base pair DNA sequence encoding a polypeptide composed of 80% essential amino acids and used it with the nopaline synthetase (NOS) promoter to construct a chimeric gene. Expression of this gene in the tuber of transgenic potato has resulted in an  
10 accumulation of this protein at a level of 0.02% to 0.35% of the total plant protein. This low level accumulation is possibly due to the weak NOS promoter and/or the instability of the new protein.

Tobacco has been used as a test plant to demonstrate the feasibility of this approach by transferring a chimeric gene containing the bean phaseolin promoter  
15 and the cDNA of a sulfur-rich protein Brazil Nut Protein ("BNP"), (18 mol% methionine and 8 mol% cysteine) into tobacco. Amino acid analysis indicates that the methionine content in the transgenic seeds is enhanced by 30% over that of the untransformed seeds. This same chimeric gene has also been transferred into a commercial crop, canola, and similar levels of enhancement were achieved.

20 However, an adverse effect is that lysine content decreases. Additionally, BNP has been identified as a major food allergen. Thus it is neither practical nor desirable to use BNP to enhance the nutritional value of crop plants.

Thus, there is a need to improve the nutritional value of plant seeds. The genetic modification should not be accompanied by detrimental side effects such as allergenicity, anti-nutritional quality or poor yield.

### **SUMMARY OF THE INVENTION**

5 It is an object of the present invention to provide a seed, the endosperm of which contains elevated levels of an essential amino acid.

It is a further object of the present invention to provide methods for increasing the nutritional value of feed.

10 It is a further object of the present invention to provide methods for genetically modifying seeds so as to increase amounts of essential amino acids which are present in relatively low amounts in unmodified seeds.

It is a further object of the present invention to provide methods for increasing the nutritional content of seeds without detrimental side effects such as allergenicity or anti-nutritional quality.

15 It is a further object of the present invention to provide methods for increasing the nutritional content of seeds while maintaining a high yield.

It is a further object of the present invention to provide a method for the expression of a polypeptide in a seed having levels of a preselected amino acid sufficient to reduce or obviate feed supplementation.

20 According to the present invention a transformed plant seed is provided, the endosperm of which is characterized as having an elevated level of at least one preselected amino acid compared to a seed from a corresponding plant which has not been transformed, wherein the amino acid is lysine, threonine, or tryptophan and optionally a sulfur-containing amino acid.

Also provided is a seed from a plant which has been transformed to express a heterologous protein in the endosperm of the seed, wherein the seed exhibits an elevated level of an essential amino acid.

5 An expression cassette is also provided comprising a seed endosperm-preferred promoter operably linked to a structural gene encoding a polypeptide having an elevated level of a preselected amino acid. Transformed plants and seeds containing the expression cassette are also provided.

10 A method for elevating the level of a preselected amino acid in the endosperm of plant seed is also provided. The method comprises the transformation of plant cells by introducing the expression cassette, recovering the transformed cells, regenerating a transformed plant and collecting the seeds therefrom.

#### **DETAILED DESCRIPTION OF THE INVENTION**

15 As used herein, a "structural gene " means an exogenous or recombinant DNA sequence or segment that encodes a polypeptide.

As used herein, "recombinant DNA" is a DNA sequence or segment that has been isolated from a cell, purified, synthesized or amplified.

As used herein, "isolated" means either physically isolated from the cell or synthesized *in vitro* on the basis of the sequence of an isolated DNA segment.

20 As used herein, the term "increased" or "elevated" levels of the preselected amino acid in a protein means that the protein contains an elevated amount of a preselected amino acid compared to the amount in an average protein.

As used herein, "increased" or "elevated" levels or amounts of preselected amino acids in a transformed plant or seed are levels which are greater than the levels or amounts in the corresponding untransformed plant or seed.

As used herein, "polypeptide" means proteins, protein fragments, modified  
5 proteins, amino acid sequences and synthetic amino acid sequences.

As used herein, "transformed plant" means a plant which comprises a structural gene which is introduced into the genome of the plant by transformation.

As used herein, "untransformed plant" refers to a wild type plant, i.e., one where the genome has not been altered by the introduction of the structural gene.

10 As used herein, "plant" includes but is not limited to plant cells, plant tissue and plant seeds.

As used herein, "seed endosperm-preferred promoter" is a promoter which preferentially promotes expression of the structural gene in the endosperm of the seed.

15 As used herein with respect to a structural gene encoding a polypeptide, the term "expresses" means that the structural gene is incorporated into the genome of cells, so that the product encoded by the structural gene is produced within the cells.

As used herein, the term "essential amino acid" means an amino acid which is synthesized only by plants or microorganisms or which is not produced by animals  
20 in sufficient quantities to support normal growth and development.

As used herein, the term "high lysine content protein" means that the protein has at least about 7 mole % lysine, preferably about 7 mole % to about 50 mole % lysine, more preferably about 7 mole % to about 40 mole % lysine and most preferably about 7 mole % to about 30 mole %.

As used herein, the term "high sulfur content protein" means that the protein contains at least about 6 mole % methionine and/or cysteine, preferably about 6 mole % to about 40 mole %, more preferably about 6 mole % to about 30 mole % and most preferably 6 mole % to 25 mole %.

5        The present invention provides a transformed plant seed, the endosperm of which is characterized as having an elevated level of a preselected amino acid compared to the seed of a corresponding plant which has not been transformed. It is preferred that the level of preselected amino acid is elevated in the endosperm in preference to other parts of the seed.

10        The preselected amino acid is an essential amino acid such as lysine, cysteine, methionine, threonine, tryptophan, arginine, valine, leucine, isoleucine, histidine or combinations thereof, preferably, the preselected amino acid is lysine, threonine, cysteine, tryptophan, or combinations thereof and optionally methionine. It is especially preferred that the polypeptide has an increased content of lysine as  
15        well as a sulfur containing amino acid, i.e., methionine and/or cysteine.

      The polypeptide can be an endogenous or heterologous protein. When an endogenous protein is expressed, the preselected amino acid is lysine, cysteine, threonine, tryptophan, arginine, valine, leucine, isoleucine, histidine or combinations thereof and optionally methionine. When the protein is a heterologous protein, any  
20        of the above described preselected amino acids or combinations thereof is present in elevated amounts.

      Generally the amount of preselected amino acid in the seed of the present invention is at least about 10 percent by weight greater than in a corresponding untransformed seed, preferably about 10 percent by weight to about 10 times

greater, more preferably about 15 percent by weight to about 10 time greater and most preferably about 20 percent to about 10 times greater.

A polypeptide having an elevated amount of the preselected amino acid is expressed in the transformed plant seed endosperm in an amount sufficient to  
5 increase the amount of at least one preselected amino acid in the seed of the transformed plant, relative to the amount of the preselected amino acid in the seed of a corresponding untransformed plant.

The choice of the structural gene is based on the desired amino acid composition of the polypeptide encoded by the structural gene, and the ability of the  
10 polypeptide to accumulate in seeds. The amino acid composition of the polypeptide can be manipulated by methods, such as site-directed mutagenesis of the structural gene encoding the polypeptide, so as to result in expression of a polypeptide that is increased in the amount of a particular amino acid. For example, site-directed mutagenesis can be used to increase levels of lysine, methionine, cysteine,  
15 threonine and/or tryptophan and/or to decrease levels of asparagine and/or glutamine.

The derivatives differ from the wild-type protein by one or more amino acid substitutions, insertions, deletions or the like. Typically, amino acid substitutions are conservative. In the regions of homology to the native sequence, variants preferably  
20 have at least 90% amino acid sequence identity, more preferably at least 95% identity.

Typical examples of suitable proteins include barley chymotrypsin inhibitor, barley alpha hordothionin, soybean 2S albumin proteins, ~~rice high methionine~~  
~~protein and sunflower high methionine protein~~ and derivatives of each protein.



Barley alpha hordothionin has been modified to increase the level of particular amino acids. The sequences of genes which express modified alpha hordothionin proteins with enhanced essential amino acids are based on the mRNA sequence of the native *Hordeum vulgare* alpha hordothionin gene (accession number X05901, Ponz *et al.* 1986 Eur. J. Biochem. 156:131-135).

*Handwritten: 7-15-97*  
~~Modified hordothionin proteins are described in U.S. Ser. Nos. 08/838,763 filed April 10, 1997; 08/824,379 filed March 26, 1997; 08/824,382 filed March 26, 1997; and U.S. Pat. No. 5,703,409 issued December 30, 1997 the disclosures of which are incorporated herein in their entirety by reference.~~

*Handwritten: 10*  
Alpha hordothionin is a 45-amino acid protein which is stabilized by four disulfide bonds resulting from eight cysteine residues. In its native form, the protein is especially rich in arginine and lysine residues, containing 5 residues (10%) of each. However, it is devoid of the essential amino acid methionine.

*Handwritten: 15*  
Alpha hordothionin has been modified to increase the amount of various amino acids such as lysine, threonine or methionine. The protein has been synthesized and the three-dimensional structure determined by computer modeling. The modeling of the protein predicts that the ten charged residues (arginine at positions 5, 10, 17, 19 and 30, and lysine at positions 1, 23, 32, 38 and 45) all occur on the surface of the molecule. The side chains of the polar amino acids (asparagine at position 11, glutamine at position 22 and threonine at position 41) also occur on the surface of the molecule. Furthermore, the hydrophobic amino acids, (such as the side chains of leucine at positions 8, 15, 24 and 33 and valine at position 18) are also solvent-accessible.

The Three-dimensional modeling of the protein indicates that the arginine residue at position 10 is important to retention of the appropriate 3-dimensional structure and possible folding through hydrogen bond interactions with the C-terminal residue of the protein. A lysine, methionine or threonine substitution at that point would disrupt this hydrogen bonding network, leading to a destabilization of the structure. The synthetic peptide having this substitution could not be made to fold correctly, which supported this analysis. Conservation of the arginine residue at position 10 provides a protein which folds correctly.

Alpha hordothionin has been modified to contain 12 lysine residues in the mature hordothionin peptide, referred to as HT12. (Rao *et al.* 1994 Protein Engineering 7(12):1485-1493 and WO 94/16078 published July 21, 1994) The disclosure of each of these is incorporated herein by reference in their entirety.

Further analysis of substitutions which would not alter the 3-dimensional structure of the molecule led to replacement of Asparagine-11, Glutamine-22 and Threonine-41 with lysine residues with virtually no steric hindrance. The resulting compound contains 27% lysine residues.

Other combinations of these substitutions were also made, including changes in amino acid residues at one or more of positions 5, 11, 17, 19, 22, 30 and 41 are lysine, and the remainder of the residues at those positions are the residues at the corresponding positions in the wild type hordothionin.

Since threonine is a polar amino acid, the surface polar amino acid residues, asparagine at position 11 and glutamine at position 22, can be substituted; and the charged amino acids, lysine at positions 1, 23, 32 and 38 and arginine at positions 5,

17, 19, and 30, can also be substituted with threonine. The molecule can be synthesized by solid phase peptide synthesis.

While the above sequence is illustrative of the present invention, it is not intended to be a limitation. Threonine substitutions can also be performed at positions containing charged amino acids. Only arginine at position 10 and lysine at position 45 are important for maintaining the structure of the protein. One can also substitute at the sites having hydrophobic amino acids. These include positions 8, 15, 18 and 24.

Since methionine is a hydrophobic amino acid, the surface hydrophobic amino acid residues, leucine at positions 8, 15, and 33, and valine at position 18, were substituted with methionine. The surface polar amino acids, asparagine at position 11, glutamine at position 22 and threonine at position 41, are substituted with methionine. The molecule is synthesized by solid phase peptide synthesis and folds into a stable structure. It has seven methionine residues (15.5%) and, including the eight cysteines, the modified protein has a sulfur amino acid content of 33%.

While the above-described proteins are illustrative of suitable polypeptides which can be expressed in the transformed plant, it is not intended to be a limitation. Methionine substitutions can also be performed at positions containing charged amino acids. Only arginine at position 10 is important for maintaining the structure of the protein through a hydrogen-bonding network with serine at position 2 and lysine at position 45. Thus, one can substitute methionine for lysine at positions 1, 23, 32, and/or 38, and for arginine at positions 5, 17, 19 and/or 30.

Many other proteins are also appropriate, for example the protein encoded by the structural gene can be a lysine and/or sulfur rich seed protein, such as the soybean 2S albumin described in U.S. Ser. No. 08/618,911 filed March 20, 1996, and the chymotrypsin inhibitor from barley, Williamson *et al.*, Eur. J Biochem 165: 99-106 (1987), the disclosures of each are incorporated by reference.

Derivatives of these genes can be made by site directed mutagenesis to increase the level of preselected amino acids in the encoded polypeptide. For example the gene encoding for the barley high lysine polypeptide (BHL), is derived from barley chymotrypsin inhibitor, U.S. Ser. No. 08/740,682 filed November 1, 1996 and PCT/US97/20441 filed October 31, 1997, the disclosures of each are incorporated herein by reference. The gene encoding for the enhanced soybean albumin gene (ESA), is derived from soybean 2S albumin described in U.S. Ser. No. 08/618,911, the disclosure of which is incorporated herein in its entirety by reference.

Other examples of sulfur-rich plant proteins within the scope of the invention include plant proteins enriched in cysteine but not methionine, such as the wheat endosperm purothionine (Mak and Jones; Can. J. Biochem.; Vol. 22; p. 83J; (1976); incorporated herein in its entirety by reference), the pea low molecular weight albumins (Higgins, *et al.*; J. Biol. Chem.; Vol. 261; p. 11124; (1986); incorporated herein in its entirety by reference) as well as 2S albumin genes from other organisms. See, for example, Coulter, *et al.*; J. Exp. Bot.; Vol. 41; p. 1541; (1990); incorporated herein in its entirety by reference.

Such proteins also include methionine-rich plant proteins ~~such as from~~  
sunflower seed (Lilley, *et al.*; In: Proceedings of the World Congress on Vegetable  
Protein Utilization in Human Foods and Animal Feedstuffs; Applewhite, H. (ed.);  
American Oil Chemists Soc.; Champaign, IL; pp. 497-502; (1989); incorporated  
5 herein in its entirety by reference), corn (Pedersen, *et al.*; J. Biol. Chem. p. 261; p.  
6279; (1986); Kirihaara, *et al.*; Gene, Vol. 71; p. 359; (1988); both incorporated herein  
in its entirety by reference), and rice (Musumura, *et al.*; Plant Mol. Biol.; Vol. 12; p.  
123; (1989); incorporated herein in its entirety by reference).

The present invention also provides a method for genetically modifying plants  
10 to increase the level of at least one preselected amino acid in the endosperm of the  
seed so as to enhance the nutritional value of the seeds.

The method comprises the introduction of an expression cassette into  
regenerable plant cells to yield transformed plant cells. The expression cassette  
comprises a seed endosperm-preferred promoter operably linked to a structural  
15 gene encoding a polypeptide elevated in content of the preselected amino acid.

A fertile transformed plant is regenerated from the transformed cells, and  
seeds are isolated from the plant. The structural gene is transmitted through a  
complete normal sexual cycle of the transformed plant to the next generation.

The polypeptide is synthesized in the endosperm of seed of the plant which  
20 has been transformed by insertion of the expression cassette described above.  
The sequence for the nucleotide molecule, either RNA or DNA, can readily be  
derived from the amino acid sequence for the selected polypeptide using standard  
reference texts.

Plants which can be used in the method of the invention include monocotyledonous cereal plants. Preferred plants include maize, wheat, rice, barley, oats, sorghum, millet and rye. The most preferred plant is maize.

Seeds derived from plants regenerated from transformed plant cells, plant parts or plant tissues, or progeny derived from the regenerated transformed plants, may be used directly as feed or food, or further processing may occur.

### **Transformation**

The transformation of plants in accordance with the invention may be carried out in essentially any of the various ways known to those skilled in the art of plant molecular biology. These include, but are not limited to, microprojectile bombardment, microinjection, electroporation of protoplasts or cells comprising partial cell walls, and *Agrobacterium*-mediated DNA transfer.

#### **I. DNA Used for Transformation**

DNA useful for introduction into plant cells includes DNA that has been derived or isolated from any source, that may be subsequently characterized as to structure, size and/or function, chemically altered, and later introduced into the plant.

An example of DNA "derived" from a source, would be a DNA sequence or segment that is identified as a useful fragment within a given organism, and which is then synthesized in essentially pure form. An example of such DNA "isolated" from a source would be a useful DNA sequence that is excised or removed from the source by chemical means, e.g., by the use of restriction endonucleases, so that it can be further manipulated, e.g., amplified, for use in the invention, by the methodology of genetic engineering.

Therefore, useful DNA includes completely synthetic DNA, semi-synthetic DNA, DNA isolated from biological sources, and DNA derived from RNA. The DNA isolated from biological sources, or DNA derived from RNA, includes, but is not limited to, DNA or RNA from plant genes, and non-plant genes such as those from bacteria, yeasts, animals or viruses. The DNA or RNA can include modified genes, portions of genes, or chimeric genes, including genes from the same or different genotype.

The term "chimeric gene" or "chimeric DNA" is defined as a gene or DNA sequence or segment comprising at least two DNA sequences or segments from species which do not recombine DNA under natural conditions, or which DNA sequences or segments are positioned or linked in a manner which does not normally occur in the native genome of untransformed plant.

A structural gene of the invention can be identified by standard methods, e.g., enrichment protocols, or probes, directed to the isolation of particular nucleotide or amino acid sequences. The structural gene can be identified by obtaining and/or screening of a DNA or cDNA library generated from nucleic acid derived from a particular cell type, cell line, primary cells, or tissue.

Screening for DNA fragments that encode all or a portion of the structural gene can be accomplished by screening plaques from a genomic or cDNA library for hybridization to a probe of the structural gene from other organisms or by screening plaques from a cDNA expression library for binding to antibodies that specifically recognize the polypeptide encoded by the structural gene.

DNA fragments that hybridize to a structural gene probe from other organisms and/or plaques carrying DNA fragments that are immunoreactive with antibodies to the polypeptide encoded by the structural gene can be subcloned into a vector and sequenced and/or used as probes to identify other cDNA or genomic sequences encoding all or a portion of the structural gene.

Portions of the genomic copy or copies of the structural gene can be partially sequenced and identified by standard methods including either DNA sequence homology to other homologous genes or by comparison of encoded amino acid sequences to known polypeptide sequences.

Once portions of the structural gene are identified, complete copies of the structural gene can be obtained by standard methods, including cloning or polymerase chain reaction (PCR) synthesis using oligonucleotide primers complementary to the structural gene. The presence of an isolated full-length copy of the structural gene can be verified by comparison of its deduced amino acid sequence with the amino acid sequence of native polypeptide sequences.

As discussed above, the structural gene encoding the polypeptide can be modified to increase the content of particular amino acid residues in that polypeptide by methods well known to the art, including, but not limited to, site-directed mutagenesis. Thus, derivatives of naturally occurring polypeptides can be made by nucleotide substitution of the structural gene so as to result in a polypeptide having a different amino acid at the position in the polypeptide which corresponds to the codon with the nucleotide substitution. The introduction of multiple amino acid changes in a polypeptide can result in a polypeptide which is significantly enriched in a preselected amino acid.



As noted above, the choice of the polypeptide encoded by the structural gene will be based on the amino acid composition of the polypeptide and its ability to accumulate in seeds. The amino acid can be chosen for its nutritional value to produce a value-added trait to the plant or plant part. Amino acids desirable for value-added traits, as well as a source to limit synthesis of an endogenous protein include, but are not limited to, lysine, threonine, tryptophan, methionine, and cysteine.

### **Expression Cassettes and Expression Vectors**

According to the present invention, a structural gene is identified, isolated, and combined with a seed endosperm-preferred promoter to provide a recombinant expression cassette.

The construction of such expression cassettes which can be employed in conjunction with the present invention are well known to those of skill in the art in light of the present disclosure. See, e.g., Sambrook, *et al.*; Molecular Cloning: A Laboratory Manual; Cold Spring Harbor, New York; (1989); Gelvin, *et al.*; Plant Molecular Biology Manual; (1990); Plant Biotechnology: Commercial Prospects and Problems, eds Prakash, *et al.*; Oxford & IBH Publishing Co.; New Delhi, India; (1993); and Heslot, *et al.*; Molecular Biology and Genetic Engineering of Yeasts; CRC Press, Inc., USA; (1992); each incorporated herein in its entirety by reference.

Preferred promoters useful in the practice of the invention are those seed endosperm-preferred promoters that allow expression of the structural gene selectively in seed endosperm to avoid any potential deleterious effects associated with the expression of the structural gene in the embryo.

It has been found that when endosperm-preferred promoters are employed, the total level of the preselected amino acid in the seed is increased compared to a seed produced by employing an embryo-preferred promoter, such as the globulin1 promoter. When the globulin1 promoter is employed, the polypeptide is expressed by the structural gene, but the total amount of the preselected amino acid is not increased.

Examples of suitable promoters include, but are not limited to, 27 kD gamma zein promoter and waxy promoter. See the following sites relating to the 27kD gamma zein promoter: Boronat,A., Martinez,M.C., Reina,M., Puigdomenech,P. and Palau,J.; Isolation and sequencing of a 28 kD glutelin-2 gene from maize: Common elements in the 5' flanking regions among zein and glutelin genes; Plant Sci. 47, 95-102 (1986) and Reina,M., Ponte,I., Guillen,P., Boronat,A. and Palau,J., Sequence analysis of a genomic clone encoding a Zc2 protein from Zea mays W64 A, Nucleic Acids Res. 18 (21), 6426 (1990). See the following site relating to the waxy promoter: Kloesgen,R.B., Gierl,A., Schwarz-Sommer,ZS. and Saedler,H., Molecular analysis of the waxy locus of Zea mays, Mol. Gen. Genet. 203, 237-244 (1986). The disclosures each of these are incorporated herein by reference in their entirety.

However, other endosperm-preferred promoters can be employed.

## II. DELIVERY OF DNA TO CELLS

The expression cassette or vector can be introduced into prokaryotic or eukaryotic cells by currently available methods which are described in the literature. See for example, Weising *et al.*, Ann. Rev. Genet. 2: 421-477 (1988). For example, the expression cassette or vector can be introduced into plant cells by methods including, but not limited to, *Agrobacterium*-mediated transformation, electroporation,

PEG poration, microprojectile bombardment, microinjection of plant cell protoplasts or embryogenic callus, silicon fiber delivery, infectious viruses or viroids such as retroviruses, the use of liposomes and the like, all in accordance with well-known procedures.

5        The introduction of DNA constructs using polyethylene glycol precipitation is described in Paszkowski *et al.*, Embo J. 3: 2717-2722 (1984). Electroporation techniques are described in Fromm *et al.*, Proc. Natl. Acad. Sci. 82: 5324 (1985). Ballistic transformation techniques are described in Klein *et al.*, Nature 327: 70-73 (1987). The disclosure of each of these is incorporated herein in its entirety by  
10    reference.

Introduction and expression of foreign genes in plants has been shown to be possible using the T-DNA of the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens*. Using recombinant DNA techniques and bacterial genetics, a wide variety of foreign DNAs can be inserted into T-DNA in *Agrobacterium*. Following  
15    infection by the bacterium containing the recombinant Ti plasmid, the foreign DNA is inserted into the host plant chromosomes, thus producing a genetically engineered cell and eventually a genetically engineered plant. A second approach is to introduce root-inducing (Ri) plasmids as the gene vectors.

*Agrobacterium tumefaciens*-mediated transformation techniques are well  
20    described in the literature. See, for example Horsch *et al.*, Science 233: 496-498 (1984), and Fraley *et al.*, Proc. Natl. Acad. Sci. 80: 4803 (1983). *Agrobacterium* transformation of maize is described in U.S. Patent No. 5,550,318. The disclosure of each of these is incorporated herein in its entirety by reference.

Other methods of transfection or transformation include (1) *Agrobacterium rhizogenes*-mediated transformation (see, e.g., Lichtenstein and Fuller In: Genetic Engineering, vol. 6, PWJ Rigby, Ed., London, Academic Press, 1987; and Lichtenstein, C. P., and Draper, J., In: DNA Cloning, Vol. II, D. M. Glover, Ed., Oxford, IRI Press, 1985). Application PCT/US87/02512 (WO 88/02405 published Apr. 7, 1988) describes the use of *A. rhizogenes* strain A4 and its Ri plasmid along with *A. tumefaciens* vectors pARC8 or pARC16 (2) liposome-mediated DNA uptake (see, e.g., Freeman *et al.*, Plant Cell Physiol. 25: 1353, 1984), (3) the vortexing method (see, e.g., Kindle, Proc. Natl. Acad. Sci., USA 87: 1228, (1990). The disclosure of each of these is incorporated herein in its entirety by reference.

DNA can also be introduced into plants by direct DNA transfer into pollen as described by Zhou *et al.*, Methods in Enzymology, 101:433 (1983); D. Hess, Intern Rev. Cytol., 107:367 (1987); Luo *et al.*, Plane Mol. Biol. Reporter, 6:165 (1988). The disclosure of each of these is incorporated herein in its entirety by reference.

Expression of polypeptide coding genes can be obtained by injection of the DNA into reproductive organs of a plant as described by Pena *et al.*, Nature, 325:274 (1987). The disclosure of which is incorporated herein in its entirety by reference.

DNA can also be injected directly into the cells of immature embryos and the rehydration of desiccated embryos as described by Neuhaus *et al.*, Theor. Appl. Genet., 75:30 (1987); and Benbrook *et al.*, in Proceedings Bio Expo 1986, Butterworth, Stoneham, Mass., pp. 27-54 (1986). The disclosure of each of these is incorporated herein in its entirety by reference.

Plant cells useful for transformation include cells cultured in suspension cultures, callus, embryos, meristem tissue, pollen, and the like.

A variety of plant viruses that can be employed as vectors are known in the art and include cauliflower mosaic virus (CaMV), geminivirus, brome mosaic virus, and tobacco mosaic virus.

Typical vectors useful for expression of genes in higher plants are well known in the art and include vectors derived from the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens* described by Rogers *et al.*, Meth. In Enzymol., 153:253-277 (1987). These vectors are plant integrating vectors in that on transformation, the vectors integrate a portion of vector DNA into the genome of the host plant. The disclosure of which is incorporated herein in its entirety by reference.

A particularly preferred vector is a plasmid, by which is meant a circular double-stranded DNA molecule which is not a part of the chromosomes of the cell. Exemplary *A. tumefaciens* vectors useful herein are plasmids pKYLX6 and pKYLX7 of Schardl *et al.*, Gene, 61:1-11 (1987) and Berger *et al.*, Proc. Natl. Acad. Sci. U.S.A., 86:8402-8406 (1989). Another useful vector herein is plasmid pBI101.2 that is available from Clontech Laboratories, Inc. (Palo Alto, CA). The disclosure of each of these is incorporated herein in its entirety by reference.

A cell in which the foreign genetic material in a vector is functionally expressed has been "transformed" by the vector and is referred to as a "transformant".

Either genomic DNA or cDNA coding the gene of interest may be used in this invention. The gene of interest may also be constructed partially from a cDNA clone and partially from a genomic clone.

When the gene of interest has been isolated, genetic constructs are made which contain the necessary regulatory sequences to provide for efficient expression of the gene in the host cell.

According to this invention, the genetic construct will contain (a) a genetic  
5 sequence coding for the polypeptide of interest and (b) one or more regulatory sequences operably linked on either side of the structural gene of interest. Typically, the regulatory sequences will be a promoter or a terminator. The regulatory sequences may be from autologous or heterologous sources.

The cloning vector will typically carry a replication origin, as well as specific  
10 genes that are capable of providing phenotypic selection markers in transformed host cells. Typically, genes conferring resistance to antibiotics or selected herbicides are used. After the genetic material is introduced into the target cells, successfully transformed cells and/or colonies of cells can be isolated by selection on the basis of these markers.

15 Typical selectable markers include genes coding for resistance to the antibiotic spectinomycin (e.g., the aada gene), the streptomycin phosphotransferase (SPT) gene coding for streptomycin resistance, the neomycin phosphotransferase (NPTII) gene encoding kanamycin or geneticin resistance, the hygromycin phosphotransferase (HPT) gene coding for hygromycin resistance.

20 Genes coding for resistance to herbicides include genes which act to inhibit the action of acetolactate synthase (ALS), in particular the sulfonylurea-type herbicides (e.g., the acetolactate synthase (ALS) genes containing mutations leading to such resistance in particular the S4 and/or Hra mutations), genes coding for resistance to herbicides which act to inhibit action of glutamine synthase, such as

phosphinothricin or basta (e.g., the *pat* or *bar* gene), or other such genes known in the art. The *bar* gene encodes resistance to the herbicide basta, and the ALS gene encodes resistance to the herbicide chlorsulfuron.

Typically, an intermediate host cell will be used in the practice of this invention to increase the copy number of the cloning vector. With an increased copy number, the vector containing the gene of interest can be isolated in significant quantities for introduction into the desired plant cells.

Host cells that can be used in the practice of this invention include prokaryotes, including bacterial hosts such as *E. coli*, *S. typhimurium*, and *Serratia marcescens*. Eukaryotic hosts such as yeast or filamentous fungi may also be used in this invention. Since these hosts are also microorganisms, it will be essential to ensure that plant promoters which do not cause expression of the polypeptide in bacteria are used in the vector.

The isolated cloning vector will then be introduced into the plant cell using any convenient transformation technique as described above.

### III. Regeneration and Analysis of Transformants

Following transformation, regeneration is involved to obtain a whole plant from transformed cells and the presence of structural gene (s) or "transgene(s)" in the regenerated plant is detected by assays. The seed derived from the plant is then tested for levels of preselected amino acids. Depending on the type of plant and the level of gene expression, introduction of the structural gene into the plant seed endosperm can enhance the level of preselected amino acids in an amount useful to supplement the nutritional quality of those seeds.

Using known techniques, protoplasts and cell or tissue culture can be regenerated to form whole fertile plants which carry and express the gene for a polypeptide according to this invention.

Accordingly, a highly preferred embodiment of the present invention is a transformed maize plant, the cells of which contain at least one copy of the DNA sequence of an expression cassette containing a gene encoding a polypeptide containing elevated amounts of an essential amino acid, such as HT12, BHL or ESA protein.

Techniques for regenerating plants from tissue culture, such as transformed protoplasts or callus cell lines, are known in the art. For example, see Phillips, *et al.*; Plant Cell Tissue Organ Culture; Vol. 1; p. 123; (1981); Patterson, *et al.*; Plant Sci.; Vol. 42; p. 125; (1985); Wright, *et al.*; Plant Cell Reports; Vol. 6; p. 83; (1987); and Barwale, *et al.*; Planta; Vol. 167; p. 473; (1986); each incorporated herein in its entirety by reference. The selection of an appropriate method is within the skill of the art.

Examples of the practice of the present invention detailed herein relate specifically to maize plants. However, the present invention is also applicable to other cereal plants. The expression vectors utilized herein are demonstrably capable of operation in cells of cereal plants both in tissue culture and in whole plants. The invention disclosed herein is thus operable in monocotyledonous species to transform individual plant cells and to achieve full, intact plants which can be regenerated from transformed plant cells and which express preselected polypeptides.



The introduced structural genes are expressed in the transformed plant cells and stably transmitted (somatically and sexually) to the next generation of cells produced. The vector should be capable of introducing, maintaining, and expressing a structural gene in plant cells. The structural gene is passed on to progeny by normal sexual transmission.

To confirm the presence of the structural gene (s) or "transgene(s)" in the regenerating plants, or seeds or progeny derived from the regenerated plant, a variety of assays can be performed. Such assays include Southern and Northern blotting; PCR; assays that detect the presence of a polypeptide product, e.g., by immunological means (ELISAs and Western blots) or by enzymatic function; plant part assays, such as leaf, seed or root assays; and also, by analyzing the phenotype of the whole regenerated plant.

Whereas DNA analysis techniques can be conducted using DNA isolated from any part of a plant, RNA will be expressed in the seed endosperm and hence it will be necessary to prepare RNA for analysis from these tissues.

PCR techniques can be used for detection and quantitation of RNA produced from introduced structural genes. In this application of PCR it is first necessary to reverse transcribe RNA into DNA, using enzymes such as reverse transcriptase, and then through the use of conventional PCR techniques amplify the DNA. In most instances PCR techniques, while useful, will not demonstrate integrity of the RNA product.

Further information about the nature of the RNA product may be obtained by Northern blotting. This technique will demonstrate the presence of an RNA species and give information about the integrity of that RNA. The presence or absence of an

RNA species can also be determined using dot or slot blot Northern hybridizations. These techniques are modifications of Northern blotting and will only demonstrate the presence or absence of an RNA species.

While Southern blotting and PCR may be used to detect the structural gene in question, they do not provide information as to whether the structural gene is being expressed. Expression may be evaluated by specifically identifying the polypeptide products of the introduced structural genes or evaluating the phenotypic changes brought about by their expression.

Assays for the production and identification of specific polypeptides may make use of physical-chemical, structural, functional, or other properties of the polypeptides. Unique physical-chemical or structural properties allow the polypeptides to be separated and identified by electrophoretic procedures, such as native or denaturing gel electrophoresis or isoelectric focusing, or by chromatographic techniques such as ion exchange or gel exclusion chromatography.

The unique structures of individual polypeptides offer opportunities for use of specific antibodies to detect their presence in formats such as an ELISA assay. Combinations of approaches may be employed with even greater specificity such as Western blotting in which antibodies are used to locate individual gene products that have been separated by electrophoretic techniques.

Additional techniques may be employed to absolutely confirm the identity of the product of interest such as evaluation by amino acid sequencing following purification. Although these are among the most commonly employed, other procedures may be additionally used.

Very frequently, the expression of a gene product is determined by evaluating the phenotypic results of its expression. These assays also may take many forms, including but not limited to, analyzing changes in the chemical composition, morphology, or physiological properties of the plant. In particular, the elevated  
5 preselected amino acid content due to the expression of structural genes encoding polypeptides can be detected by amino acid analysis.

Breeding techniques useful in the present invention are well known in the art.

The present invention will be further described by reference to the following detailed examples. It is understood, however, that there are many extensions,  
10 variations, and modifications on the basic theme of the present invention beyond that shown in the examples and description, which are within the spirit and scope of the present invention.

### Examples

#### **EXAMPLE 1**

15 ***Construction of the HT12 gene and of other genes encoding polypeptides having an elevated level of a preselected amino acid.***

As noted above, the sequence of the HT12 gene is based on the mRNA sequence of the native *Hordeum vulgare* alpha hordothionin gene (accession number X05901, Ponz *et al.* 1986 Eur. J. Biochem. 156:131-135) modified to  
20 introduce 12 lysine residues into the mature hordothionin peptide (See Rao *et al.* 1994 Protein Engineering 7(12):1485-1493, and WO 94/16078 published July 21, 1994).

The alpha hordothionin cDNA comprising the entire alpha hordothionin coding sequence is isolated by rt-PCR of mRNA from developing barley seed. Primers are

designed based upon the published alpha hordothionin sequence to amplify the gene and to introduce a NcoI site at the start (ATG) codon and a BamHI site after the stop codon of the thionin coding sequence to facilitate cloning.

Sub 18 Primers are designated as HTPCR1 (5'-  
5 AGTATAAGTAAACACACCATCACACCCTTGAGGCCCTTGCTGGTGGCCATGGT  
G-3') and HTPCR2 (5'-  
CCTCACATCCCTTAGTGCCTAAGTTCGACGTCGGGCCCTCTAGTCGACGGATC  
CA-3'). These primers are used in a PCR reaction to amplify alpha hordothionin by  
conventional methods. The resulting PCR product is purified and subcloned into the  
10 BamHI/NcoI digested pBSKP vector (Stratagene, LaJolla, CA) and sequenced on  
both strands to confirm its identity. The clone is designated pBSKP-HT (seq. ID 1).  
Primers are designed for single stranded DNA site-directed mutagenesis to  
introduce 12 codons for lysine, based on the peptide structure of hordothionin 12  
(Ref: Rao *et al.* 1994 Protein Engineering 7(12):1485-1493) and are designated  
15 HT12mut1 (5'-AGCGGAAAATGCCCGAAAGGCTTCCCCAAATTGGC-3'),  
HT12mut2 (5'-  
TGCGCAGGCGTCTGCAAGTGTAAGCTGACTAGTAGCGGAAAATGC-3'),  
HT12mut3 (5'-  
TACAACCTTTGCAAAGTCAAAGGCGCCAAGAAGCTTTGCGCAGGCGTCTG-3'),  
20 HT12mut4 (5'-  
GCAAGAGTTGCTGCAAGAGTACCCTGGGAAGGAAGTGCTACAACCTTTGC-3').

Sequence analysis is used to verify the desired sequence of the resulting plasmid, designated pBSKP-HT12 (seq. ID 2).

Similarly, genes encoding other derivatives of hordothionin, as described above, (See U.S. Ser. Nos. 08/838,763 filed April 10, 1997; ~~08/824,379~~ filed March

~~26, 1997; 08/824,382~~ filed March 26, 1997; and U.S. Pat. No. 5,703,409 issued

December 30, 1997), the gene encoding enhanced soybean albumin (ESA) (See

U.S. Ser. No. 08/618,911), and genes encoding BHL and other derivatives of the

barley chymotrypsin inhibitor (See U.S. Ser. No. 08/740,682 filed November 1, 1996

and PCT/US97/20441 filed October 31, 1997) are constructed by site directed

mutagenesis from pBSKP-HT, a subclone of the soybean 2S albumin 3 gene in the

pBSKP vector (Stratagene, LaJolla, CA), and a subclone of the barley chymotrypsin

inhibitor in the pBSKP vector, respectively.

## **EXAMPLE 2**

***Construction of vectors for seed preferred expression of polypeptides having an elevated level of a preselected amino acid.***

A 442bp DNA fragment containing the modified hordothionin gene encoding HT12 is isolated from plasmid pBSKP-HT12 by NcoI/BamHI restriction digestion, gel purification and is ligated between the 27 kD gamma zein promoter and 27kD gamma zein terminator of the NcoI/BamHI digested vector PHP3630. PHP 3630 is a subclone of the endosperm-preferred 27kD gamma zein gene (Genbank accession number X58197) in the pBSKP vector (Stratagene), which is modified by site directed mutagenesis by insertion of a NcoI site at the start codon (ATG) of the 27kD gamma zein coding sequence. The 27kD gamma zein coding sequence is replaced with the HT12 coding sequence. The resulting expression vector containing the chimeric gene construct gz::HT12::gz, designated as PHP8001 (Seq. ID 3), is verified by extensive restriction digest analysis and DNA sequencing.

Similarly, the 442bp DNA fragment containing the HT12 coding sequence is inserted between the globulin1 promoter and the globulin1 terminator of the embryo preferred corn globulin1 gene (Genbank accession number X59083), and between the waxy promoter and the waxy terminator of the endosperm-preferred waxy gene (Genbank accession number M24258). The globulin1 and waxy coding sequences, respectively, are replaced with the HT12 coding sequence. The resulting chimeric genes glb1::HT12::glb1, and wx::HT12::wx are designated as PHP 7999 (Seq. ID 4), and PHP 5025 (Seq. ID 5).

Sub 15  
10 In a like manner, expression vectors containing genes encoding other derivatives of hordothionin (See Rao *et al.*, 1994 Protein Engineering 7(12):1485-1493, and WO 94/16078 published July 21, 1994), the gene encoding enhanced soybean albumin (ESA) (See U.S. Ser. No. 08/618,911,), and genes encoding BHL and other derivatives of the barley chymotrypsin inhibitor (See U.S. Ser. No. 08/740,682 filed November 1, 1996 and PCT/US97/20441 filed October 31, 1997) are constructed by insertion of the corresponding coding sequences between the promoter and terminator of the 27kD gamma zein gene, the globulin1 gene and the waxy gene, respectively. Resulting chimeric genes are for example gz::ESA::gz and gz::BHL::gz, designated as PHP11260 (Seq. ID 6) and as PHP11427 (Seq. ID 7), respectively.

20 The resulting expression vectors are used in conjunction with the selectable marker expression cassettes PHP3528 (enhanced CAMV::Bar::PinII) for particle bombardment transformation of maize immature embryos.

### **EXAMPLE 3**

#### ***Preparation of Transgenic Plants***

The general method of genetic transformation used to produce transgenic maize plants is mediated by bombardment of embryogenically responsive immature embryos with tungsten particles associated with DNA plasmids, said plasmids consisting of a selectable and an unselectable marker gene.

#### **Preparation of Tissue**

Immature embryos of "High Type II" are the target for particle bombardment-mediated transformation. This genotype is the  $F_1$  of two purebred genetic lines, parent A and parent B, derived from A188 X B73. Both parents are selected for high competence of somatic embryogenesis. See Armstrong, *et al.*, "Development and Availability of Germplasm with High Type II Culture Formation Response," Maize Genetics Cooperation Newsletter, Vol. 65, pp. 92 (1991); incorporated herein in its entirety by reference.

Ears from  $F_1$  plants are selfed or sibbed, and embryos are aseptically dissected from developing caryopses when the scutellum first becomes opaque. The proper stage occurs about 9-13 days post-pollination, and most generally about 10 days post-pollination, and depends on growth conditions. The embryos are about 0.75 to 1.5 mm long. Ears are surface sterilized with 20-50% Clorox for 30 min, followed by 3 rinses with sterile distilled water.

Immature embryos are cultured, scutellum oriented upward, on embryogenic induction medium comprised of N6 basal salts (Chu, *et al.*, "Establishment of an Efficient Medium for Anther Culture of Rice Through Comparative Experiments on the Nitrogen Sources," Scientia Sinica, (Peking), Vol. 18, pp. 659-668 (1975);

incorporated herein in its entirety by reference; Eriksson vitamins (See Eriksson, T., "Studies on the Growth Requirements and Growth Measurements of Haplopappus gracilis," Physiol. Plant, Vol. 18, pp. 976-993 (1965); incorporated herein in its entirety by reference), 0.5 mg/l thiamine HCl, 30 gm/l sucrose, 2.88 gm/l L-proline, 1  
5 mg/l 2,4-dichlorophenoxyacetic acid, 2 gm/l Gelrite, and 8.5 mg/l AgNO<sub>3</sub>.

The medium is sterilized by autoclaving at 121°C for 15 min and dispensed into 100 X 25 mm petri dishes. AgNO<sub>3</sub> is filter-sterilized and added to the medium after autoclaving. The tissues are cultured in complete darkness at 28°C. After about 3 to 7 days, generally about 4 days, the scutellum of the embryo has swelled  
10 to about double its original size and the protuberances at the coleorhizal surface of the scutellum indicate the inception of embryogenic tissue. Up to 100% of the embryos display this response, but most commonly, the embryogenic response frequency is about 80%.

When the embryogenic response is observed, the embryos are transferred to  
15 a medium comprised of induction medium modified to contain 120 gm/l sucrose. The embryos are oriented with the coleorhizal pole, the embryogenically responsive tissue, upwards from the culture medium. Ten embryos per petri dish are located in the center of a petri dish in an area about 2 cm in diameter. The embryos are maintained on this medium for 3-16 hr, preferably 4 hours, in complete darkness at  
20 28°C just prior to bombardment with particles associated with plasmid DNAs containing the selectable and unselectable marker genes.

To effect particle bombardment of embryos, the particle-DNA agglomerates are accelerated using a DuPont PDS-1000 particle acceleration device. The particle-DNA agglomeration is briefly sonicated and 10 µl are deposited on



macrocarriers and the ethanol allowed to evaporate. The macrocarrier is accelerated onto a stainless-steel stopping screen by the rupture of a polymer diaphragm (rupture disk). Rupture is effected by pressurized helium. Depending on the rupture disk breaking pressure, the velocity of particle-DNA acceleration may be varied. Rupture disk pressures of 200 to 1800 psi are commonly used, with those of 650 to 1100 psi being more preferred, and about 900 psi being most highly preferred. Rupture disk breaking pressures are additive so multiple disks may be used to effect a range of rupture pressures.

Preferably, the shelf containing the plate with embryos is 5.1 cm below the bottom of the macrocarrier platform (shelf #3), but may be located at other distances. To effect particle bombardment of cultured immature embryos, a rupture disk and a macrocarrier with dried particle-DNA agglomerates are installed in the device. The He pressure delivered to the device is adjusted to 200 psi above the rupture disk breaking pressure. A petri dish with the target embryos is placed into the vacuum chamber and located in the projected path of accelerated particles. A vacuum is created in the chamber, preferably about 28 inches Hg. After operation of the device, the vacuum is released and the petri dish is removed.

Bombarded embryos remain on the osmotically adjusted medium during bombardment, and preferably for two days subsequently, although the embryos may remain on this medium for 1 to 4 days. The embryos are transferred to selection medium comprised of N6 basal salts, Eriksson vitamins, 0.5 mg/l thiamine HCl, 30 gm/l sucrose, 1 mg/l 2,4-dichlorophenoxyacetic acid, 2 gm/l Gelrite, 0.85 mg/l AgNO<sub>3</sub> and 3 mg/l bialaphos. Bialaphos is added filter-sterilized. The embryos are subcultured to fresh selection medium at 10 to 14 day intervals. After about 7 weeks,

embryogenic tissue, putatively transgenic for both selectable and unselected marker genes, is seen to proliferate from about 7% of the bombarded embryos. Putative transgenic tissue is rescued, and that tissue derived from individual embryos is considered to be an event and is propagated independently on selection medium.

- 5 Two cycles of clonal propagation is achieved by visual selection for the smallest contiguous fragments of organized embryogenic tissue.

For regeneration of transgenic plants, embryogenic tissue is subcultured to medium comprised of MS salts and vitamins (Murashige, T. and F. Skoog, "A revised medium for rapid growth and bio assays with tobacco tissue cultures";  
10 Physiologia Plantarum; Vol. 15; pp. 473-497; 1962; incorporated herein in its entirety by reference), 100 mg/l myo-inositol, 60 gm/l sucrose, 3 gm/l Gelrite, 0.5 mg/l zeatin, 1 mg/l indole-3-acetic acid, 26.4 ng/l cis-trans-abscissic acid, and 3 mg/l bialaphos in 100 X 25 mm petri dishes and incubated in darkness at 28°C until the development of well-formed, matured somatic embryos can be visualized. This requires about 14  
15 days.

Well-formed somatic embryos are opaque and cream-colored, and are comprised of an identifiable scutellum and coleoptile. The embryos are individually subcultured to germination medium comprised of MS salts and vitamins, 100 mg/l myo-inositol, 40 gm/l sucrose and 1.5 gm/l Gelrite in 100 X 25 mm petri dishes and  
20 incubated under a 16 hr light: 8 hr dark photoperiod and 40  $\mu\text{Einsteins m}^{-2}\text{sec}^{-1}$  from cool-white fluorescent tubes. After about 7 days, the somatic embryos have germinated and produced a well-defined shoot and root. The individual plants are subcultured to germination medium in 125 x 25 mm glass tubes to allow further plant

development. The plants are maintained under a 16 hr light: 8 hr dark photoperiod and  $40 \mu\text{Einsteins m}^{-2}\text{sec}^{-1}$  from cool-white fluorescent tubes.

After about 7 days, the plants are well-established and are transplanted to horticultural soil, hardened off, and potted into commercial greenhouse soil mixture and grown to sexual maturity in a greenhouse. An elite inbred line is used as a male to pollinate regenerated transgenic plants.

#### Preparation of Particles

Fifteen mg of tungsten particles (General Electric) , 0.5 to 1.8  $\mu\text{m}$ , preferably 1 to 1.8  $\mu\text{m}$ , and most preferably 1  $\mu\text{m}$ , are added to 2 ml of concentrated nitric acid. This suspension is sonicated at  $0^{\circ}\text{C}$  for 20 min (Branson Sonifier Model 450, 40% output, constant duty cycle). Tungsten particles are pelleted by centrifugation at 10,000 rpm (Biofuge) for 1 min and the supernatant is removed. Two ml of sterile distilled water is added to the pellet and sonicate briefly to resuspend the particles. The suspension is pelleted, 1 ml of absolute ethanol is added to the pellet and sonicated briefly to resuspend the particles. Rinse, pellet, and resuspend the particles a further 2 times with sterile distilled water, and finally resuspend the particles in 2 ml of sterile distilled water. The particles are subdivided into 250  $\mu\text{l}$  aliquots and stored frozen.

#### Preparation of particle-plasmid DNA association

The stock of tungsten particles is sonicated briefly in a water bath sonicator (Branson Sonifier Model 450, 20% output, constant duty cycle) and 50  $\mu\text{l}$  is transferred to a microfuge tube. Plasmid DNA is added to the particles for a final DNA amount of 0.1 to 10  $\mu\text{g}$  in 10  $\mu\text{l}$  total volume, and briefly sonicated. Preferably 1  $\mu\text{g}$  total DNA is used. Specifically, 5  $\mu\text{l}$  of PHP8001 (gz::HT12::gz) and 5  $\mu\text{l}$  of

PHP3528 (enhanced CAMV::Bar::PinII) at 0.1  $\mu\text{g}/\mu\text{l}$  in TE buffer, are added to the particle suspension. Fifty  $\mu\text{l}$  of sterile aqueous 2.5 M  $\text{CaCl}_2$  are added, and the mixture is briefly sonicated and vortexed. Twenty  $\mu\text{l}$  of sterile aqueous 0.1M spermidine are added and the mixture is briefly sonicated and vortexed. The mixture is incubated at room temperature for 20 min with intermittent brief sonication. The particle suspension is centrifuged, and the supernatant is removed. Two hundred fifty  $\mu\text{l}$  of absolute ethanol is added to the pellet and briefly sonicated. The suspension is pelleted, the supernatant is removed, and 60  $\mu\text{l}$  of absolute ethanol is added. The suspension is sonicated briefly before loading the particle-DNA agglomeration onto macrocarriers.

#### **EXAMPLE 4**

***Analysis of seed from transgenic plants for recombinant polypeptides having an elevated level of a preselected amino acid.***

##### **Preparation of meals from corn seed**

Pooled or individual dry seed harvested from transformed plants from the greenhouse or the field are prepared in one of the following ways:

- A. Seed is imbibed in sterile water overnight (16-20 hr) at 4°C. The imbibed seed is dissected into embryo, endosperm and pericarp. The embryos and endosperm are separately frozen in liquid  $\text{N}_2$ , the pericarps are discarded. Frozen tissue is ground with a liquid  $\text{N}_2$  chilled ceramic mortar and pestle to a fine meal. The meals are dried under vacuum and stored at -20°C or -80°C.

B. Dry whole seed is ground to a fine meal with a ball mill (Klecko), or by hand with a ceramic mortar and pestle. For analysis of endosperm only, the embryos are removed with a drill and discarded. The remaining endosperm with pericarp is ground with a ball mill or a mortar and pestle.

5 ELISA analysis

Rabbit polyclonal anti HT12 antisera are produced with synthetic HT12 (See Rao *et al. supra*) at Bethyl laboratories. An HT12 ELISA assay is developed and performed by the Analytical Biochemistry department of Pioneer Hi-Bred International, Inc., essentially as described by Harlow and Lane, Antibodies, A Laboratory Manual, Cold Springs Harbor Publication, New York (1988). Quantitative ELISA assays are first performed on pooled meals to identify positive events. Positive events are further analyzed by quantitative ELISA on individual kernels to determine the relative level of HT12 expression and transgene segregation ratio. Among 97 events tested, 59 show HT12 expression levels >1000 ppm. The highest events have HT12 expression levels at 2-5% of the total seed protein. Typical results for HT12 levels for whole kernels of wild type corn, for one event (TC2031) of corn transformed with the *gz::HT12::gz* chimeric gene, expressing HT12 in the endosperm, for one event (TC320) of corn transformed with the *wx::HT12::wx* chimeric gene, expressing HT12 in the endosperm, and for one event (TC2027) of corn transformed with the *glb1::HT12::glb1* chimeric gene, expressing HT12 in the embryo, are in Table 1.

Similarly, antisera are produced, ELISA assays are developed and assays of seed from transformed plants are performed for other derivatives of hordothionine (See Rao *et al.* 1994 Protein Engineering 7(12):1485-1493, and WO 94/16078 published July 21, 1994), for the enhanced soybean albumin (ESA) (See U.S. Ser.

10  
20  
30  
40  
50  
60  
70  
80  
90  
100  
110  
120  
130  
140  
150  
160  
170  
180  
190  
200  
210  
220  
230  
240  
250  
260  
270  
280  
290  
300  
310  
320  
330  
340  
350  
360  
370  
380  
390  
400  
410  
420  
430  
440  
450  
460  
470  
480  
490  
500  
510  
520  
530  
540  
550  
560  
570  
580  
590  
600  
610  
620  
630  
640  
650  
660  
670  
680  
690  
700  
710  
720  
730  
740  
750  
760  
770  
780  
790  
800  
810  
820  
830  
840  
850  
860  
870  
880  
890  
900  
910  
920  
930  
940  
950  
960  
970  
980  
990  
1000  
1010  
1020  
1030  
1040  
1050  
1060  
1070  
1080  
1090  
1100  
1110  
1120  
1130  
1140  
1150  
1160  
1170  
1180  
1190  
1200  
1210  
1220  
1230  
1240  
1250  
1260  
1270  
1280  
1290  
1300  
1310  
1320  
1330  
1340  
1350  
1360  
1370  
1380  
1390  
1400  
1410  
1420  
1430  
1440  
1450  
1460  
1470  
1480  
1490  
1500  
1510  
1520  
1530  
1540  
1550  
1560  
1570  
1580  
1590  
1600  
1610  
1620  
1630  
1640  
1650  
1660  
1670  
1680  
1690  
1700  
1710  
1720  
1730  
1740  
1750  
1760  
1770  
1780  
1790  
1800  
1810  
1820  
1830  
1840  
1850  
1860  
1870  
1880  
1890  
1900  
1910  
1920  
1930  
1940  
1950  
1960  
1970  
1980  
1990  
2000  
2010  
2020  
2030  
2040  
2050  
2060  
2070  
2080  
2090  
2100  
2110  
2120  
2130  
2140  
2150  
2160  
2170  
2180  
2190  
2200  
2210  
2220  
2230  
2240  
2250  
2260  
2270  
2280  
2290  
2300  
2310  
2320  
2330  
2340  
2350  
2360  
2370  
2380  
2390  
2400  
2410  
2420  
2430  
2440  
2450  
2460  
2470  
2480  
2490  
2500  
2510  
2520  
2530  
2540  
2550  
2560  
2570  
2580  
2590  
2600  
2610  
2620  
2630  
2640  
2650  
2660  
2670  
2680  
2690  
2700  
2710  
2720  
2730  
2740  
2750  
2760  
2770  
2780  
2790  
2800  
2810  
2820  
2830  
2840  
2850  
2860  
2870  
2880  
2890  
2900  
2910  
2920  
2930  
2940  
2950  
2960  
2970  
2980  
2990  
3000  
3010  
3020  
3030  
3040  
3050  
3060  
3070  
3080  
3090  
3100  
3110  
3120  
3130  
3140  
3150  
3160  
3170  
3180  
3190  
3200  
3210  
3220  
3230  
3240  
3250  
3260  
3270  
3280  
3290  
3300  
3310  
3320  
3330  
3340  
3350  
3360  
3370  
3380  
3390  
3400  
3410  
3420  
3430  
3440  
3450  
3460  
3470  
3480  
3490  
3500  
3510  
3520  
3530  
3540  
3550  
3560  
3570  
3580  
3590  
3600  
3610  
3620  
3630  
3640  
3650  
3660  
3670  
3680  
3690  
3700  
3710  
3720  
3730  
3740  
3750  
3760  
3770  
3780  
3790  
3800  
3810  
3820  
3830  
3840  
3850  
3860  
3870  
3880  
3890  
3900  
3910  
3920  
3930  
3940  
3950  
3960  
3970  
3980  
3990  
4000  
4010  
4020  
4030  
4040  
4050  
4060  
4070  
4080  
4090  
4100  
4110  
4120  
4130  
4140  
4150  
4160  
4170  
4180  
4190  
4200  
4210  
4220  
4230  
4240  
4250  
4260  
4270  
4280  
4290  
4300  
4310  
4320  
4330  
4340  
4350  
4360  
4370  
4380  
4390  
4400  
4410  
4420  
4430  
4440  
4450  
4460  
4470  
4480  
4490  
4500  
4510  
4520  
4530  
4540  
4550  
4560  
4570  
4580  
4590  
4600  
4610  
4620  
4630  
4640  
4650  
4660  
4670  
4680  
4690  
4700  
4710  
4720  
4730  
4740  
4750  
4760  
4770  
4780  
4790  
4800  
4810  
4820  
4830  
4840  
4850  
4860  
4870  
4880  
4890  
4900  
4910  
4920  
4930  
4940  
4950  
4960  
4970  
4980  
4990  
5000  
5010  
5020  
5030  
5040  
5050  
5060  
5070  
5080  
5090  
5100  
5110  
5120  
5130  
5140  
5150  
5160  
5170  
5180  
5190  
5200  
5210  
5220  
5230  
5240  
5250  
5260  
5270  
5280  
5290  
5300  
5310  
5320  
5330  
5340  
5350  
5360  
5370  
5380  
5390  
5400  
5410  
5420  
5430  
5440  
5450  
5460  
5470  
5480  
5490  
5500  
5510  
5520  
5530  
5540  
5550  
5560  
5570  
5580  
5590  
5600  
5610  
5620  
5630  
5640  
5650  
5660  
5670  
5680  
5690  
5700  
5710  
5720  
5730  
5740  
5750  
5760  
5770  
5780  
5790  
5800  
5810  
5820  
5830  
5840  
5850  
5860  
5870  
5880  
5890  
5900  
5910  
5920  
5930  
5940  
5950  
5960  
5970  
5980  
5990  
6000  
6010  
6020  
6030  
6040  
6050  
6060  
6070  
6080  
6090  
6100  
6110  
6120  
6130  
6140  
6150  
6160  
6170  
6180  
6190  
6200  
6210  
6220  
6230  
6240  
6250  
6260  
6270  
6280  
6290  
6300  
6310  
6320  
6330  
6340  
6350  
6360  
6370  
6380  
6390  
6400  
6410  
6420  
6430  
6440  
6450  
6460  
6470  
6480  
6490  
6500  
6510  
6520  
6530  
6540  
6550  
6560  
6570  
6580  
6590  
6600  
6610  
6620  
6630  
6640  
6650  
6660  
6670  
6680  
6690  
6700  
6710  
6720  
6730  
6740  
6750  
6760  
6770  
6780  
6790  
6800  
6810  
6820  
6830  
6840  
6850  
6860  
6870  
6880  
6890  
6900  
6910  
6920  
6930  
6940  
6950  
6960  
6970  
6980  
6990  
7000  
7010  
7020  
7030  
7040  
7050  
7060  
7070  
7080  
7090  
7100  
7110  
7120  
7130  
7140  
7150  
7160  
7170  
7180  
7190  
7200  
7210  
7220  
7230  
7240  
7250  
7260  
7270  
7280  
7290  
7300  
7310  
7320  
7330  
7340  
7350  
7360  
7370  
7380  
7390  
7400  
7410  
7420  
7430  
7440  
7450  
7460  
7470  
7480  
7490  
7500  
7510  
7520  
7530  
7540  
7550  
7560  
7570  
7580  
7590  
7600  
7610  
7620  
7630  
7640  
7650  
7660  
7670  
7680  
7690  
7700  
7710  
7720  
7730  
7740  
7750  
7760  
7770  
7780  
7790  
7800  
7810  
7820  
7830  
7840  
7850  
7860  
7870  
7880  
7890  
7900  
7910  
7920  
7930  
7940  
7950  
7960  
7970  
7980  
7990  
8000  
8010  
8020  
8030  
8040  
8050  
8060  
8070  
8080  
8090  
8100  
8110  
8120  
8130  
8140  
8150  
8160  
8170  
8180  
8190  
8200  
8210  
8220  
8230  
8240  
8250  
8260  
8270  
8280  
8290  
8300  
8310  
8320  
8330  
8340  
8350  
8360  
8370  
8380  
8390  
8400  
8410  
8420  
8430  
8440  
8450  
8460  
8470  
8480  
8490  
8500  
8510  
8520  
8530  
8540  
8550  
8560  
8570  
8580  
8590  
8600  
8610  
8620  
8630  
8640  
8650  
8660  
8670  
8680  
8690  
8700  
8710  
8720  
8730  
8740  
8750  
8760  
8770  
8780  
8790  
8800  
8810  
8820  
8830  
8840  
8850  
8860  
8870  
8880  
8890  
8900  
8910  
8920  
8930  
8940  
8950  
8960  
8970  
8980  
8990  
9000  
9010  
9020  
9030  
9040  
9050  
9060  
9070  
9080  
9090  
9100  
9110  
9120  
9130  
9140  
9150  
9160  
9170  
9180  
9190  
9200  
9210  
9220  
9230  
9240  
9250  
9260  
9270  
9280  
9290  
9300  
9310  
9320  
9330  
9340  
9350  
9360  
9370  
9380  
9390  
9400  
9410  
9420  
9430  
9440  
9450  
9460  
9470  
9480  
9490  
9500  
9510  
9520  
9530  
9540  
9550  
9560  
9570  
9580  
9590  
9600  
9610  
9620  
9630  
9640  
9650  
9660  
9670  
9680  
9690  
9700  
9710  
9720  
9730  
9740  
9750  
9760  
9770  
9780  
9790  
9800  
9810  
9820  
9830  
9840  
9850  
9860  
9870  
9880  
9890  
9900  
9910  
9920  
9930  
9940  
9950  
9960  
9970  
9980  
9990  
10000

No. 08/618,911) and for BHL and other derivatives of the barley chymotrypsin inhibitor (See U.S. Ser. No. 08/740,682 filed November 1, 1996 and PCT/US97/20441 filed October 31, 1997), respectively.

#### Polyacrylamide gel and immuno blot analysis

5 SDS extracts of meals, molecular weight markers, and a synthetic HT12 positive control (see Rao *et al. supra*) are separated on 16.5% or 8-22% polyacrylamide gradient Tris-Tricine gels (Schagger, H. and Von Jagow, G. 1987 Anal. Biochem., 166:368). For immuno blot analysis, gels are transferred to PVDF membranes in 100 mM CAPS, pH 11; 10% methanol using a semidry blotter (Hoefer, San Francisco, CA).

10 After transfer the membrane is blocked in BLOTTO (4% dry milk in Tris-buffered saline, pH 7.5) (Johnson, D. A. , Gausch, J. W., Sportsman, J. R., and Elder, J. H. 1984, Gene Anal. Techn., 1:3). The blots are incubated with rabbit anti-HT12 (same as used for ELISA) diluted 1:2000 to 1:7500 in BLOTTO 2 hr at room temperature (22°C) or overnight at 4°C. Blots are washed 4-5X with BLOTTO, then incubated 1-2

15 hr with horseradish peroxidase-goat anti-rabbit IgG (Promega, Madison, WI) diluted 1:7500 to 1:15000 in BLOTTO. After secondary antibody, the blots are washed 3X with BLOTTO followed by 2 washes with Tris-buffered saline, pH 7.5. Blots are briefly incubated with enhanced chemiluminescence (ECL, Amersham, Arlington Heights, IL) substrate, and wrapped in plastic wrap. Reactive bands are visualized after exposure

20 to x-ray film (Kodak Biomax MR) after short exposure times ranging from 5-120 sec.

HT12 transgenic seed shows a distinctive band not seen in wild type seed at the correct molecular weight and position as judged by the HT12 positive control standard and molecular weight markers. These results indicate that the expressed HT12 prepropeptide is being correctly processed like native HT in barley. Novel polypeptide

bands co-migrating with the HT12 positive control are also observed in Coomassie stained polyacrylamide gels loaded with 10mg total extracted protein indicating substantial expression and accumulation of HT12 protein in the seed.

5 Similarly, other derivatives of hordothionin, soybean albumin, the enhanced soybean albumin (ESA), BHL and other derivatives of the barley chymotrypsin inhibitor are detected by polyacrylamide gel and immuno blot analysis.

#### Amino acid composition analysis

10 Meals from seed, endosperm or embryo that express a recombinant polypeptide having an elevated level of a preselected amino acid are sent to the University of Iowa Protein Structure Facility for amino acid composition analysis using standard protocols for digestion and analysis.

15 Typical results for the amino acid composition of whole kernels of wild type corn, for one event (TC2031) of corn transformed with the gz::HT12::gz chimeric gene, expressing HT12 in the endosperm, for one event (TC320) of corn transformed with the wx::HT12::wx chimeric gene, expressing HT12 in the endosperm, and for one event (TC2027) of corn transformed with the glb1::HT12::glb1 chimeric gene, expressing HT12 in the embryo, are in Table 1.

**Table 1:** HT12 ELISA analysis and amino acid composition of meal from whole kernels from wild type corn and from transformed corn expressing recombinant HT12.

transgene none		wx::HT12::wx	gz::HT12::gz	glb1::HT12::glb1
event	wild-type	TC320	TC2031	TC2027
ELISA				
HT 12	protein ppm 0.00	protein ppm 6200	protein ppm 8000	protein ppm 22600
AA				
	Meal % n=3	Meal % n=2	Meal % n=3	Meal % n=4
Lys	0.29	0.38	0.39	0.24
Arg	0.52	0.58	0.56	0.45
Cys	0.12	0.19	0.17	0.22

5 The results in Table 1 demonstrate corn expressing recombinant HT12 in the endosperm shows a significant increase of the preselected amino acid lysine.

**Table 2:** SEQUENCE INFORMATION

SEQUENCE ID	PROMOTER	GENE
Seq. 1: pBSKP-HT	None	3361-2947
Seq. 2: pBSKP-HT12	None	3361-2947
Seq. 3: PHP8001gz::HT12::gz expression vector	676-2198	2199-2612
Seq. 4: PHP7999 glb1::HT12::glb1 expression vector	3271-1834	1834-1420
Seq. 5: PHP5025 wx::HT::wx expression vector	43-1342	1343-1757
Seq. 6: PHP 11260 gz::ESA::gz expression vector	676-2198	2199-2675
Seq. 7: PHP11427 gz::BHL::gz	676-2198	2199-2450